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Evaluation of the Antioxidant Activities of Natural Components of *Artemisia iwayomogi*

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Abstract – The antioxidant activities of 29 components isolated from the aerial parts of *Artemisia iwayomogi* were evaluated *in vitro* and in cell culture. Among the tested compounds, **2**, **6**, **8**, **10**, **13**, and **14** exhibited the greatest peroxyl radical-scavenging activities in the oxygen radical absorbance capacity (ORAC) assay, and **2**, **10**, and **14** also showed significant reducing capacities. However, all compounds showed weak metal chelating activities. Their cellular antioxidant activities were evaluated in HepG2 cells. At 10 μM, compounds **6**, **8**, and **14** exhibited stronger protection against 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative stress than compounds **2**, **10**, and **13**. Moreover, Compounds **2** and **8** were more effective in protecting against Cu^{2+}-induced oxidative stress than compounds **6**, **10**, **13**, and **14** at 10 μM. These results suggest that the phenolic compounds in *A. iwayomogi* have the potential to be developed as natural antioxidants for the treatment of oxidative stress-related diseases.

Keywords – *Artemisia iwayomogi*, Compositae, Oxygen radical absorbance capacity (ORAC), Cellular antioxidant activity, HepG2 cell

Introduction

Oxidative stress is caused by an imbalance between the generation of reactive oxygen species (ROS) and antioxidant defense activity. Severe oxidative stress has been implicated in many chronic and degenerative diseases, including cancer, ageing, osteoporosis, rheumatoid arthritis, diabetes, and neurodegenerative diseases,[1–4] due to the damage of lipids, proteins, and DNA in living cells.[4,5] Many natural compounds from plants, including medicinal herbs, have demonstrated antioxidant activity against ROS.

Artemisia iwayomogi Kitamura is a perennial herb of the Compositae family that is distributed throughout Korea. In traditional Korean medicine, the aerial parts of *A. iwayomogi*, called "Han In Jin", have been used to cure various infectious diseases, such as carbuncle, sores, cholecystitis, and hepatitis, and to treat fever, inflammation, and jaundice.[6,7] We previously isolated 29 compounds from a 70% MeOH extract of *A. iwayomogi*, which were identified as benzoic acid (**1**), *trans*-caffeic acid methyl ester (**2**), coniferin (**3**), citrusin C (**4**), isotachioside (**5**), myrciaphenone A (**6**), 2,4-dihydroxy-6-methoxyacetophenone 4-*O*-*β*-D-glucopyranoside (**7**), 2,4-dihydroxy-6-methoxyacetophenone (**8**), erythroxyloside B (**9**), scopoletin (**10**), scopolin (**11**), fraxidin 8-*O*-*β*-D-glucopyranoside (**12**), iwayomin (**13**), methyl 3,5-di-*O*-caffeoyl quinate (**14**), (−)-syringaresinol-4-*O*-*β*-D-glucopyranoside (**15**), iwayoside A (**16**), oplodiol 1-*O*-*β*-D-glucopyranoside (**17**), iwayoside C (**18**), oplopanone 10-*O*-*β*-D-glucopyranoside (**19**), (*Z*)-5'-hydroxyjasmone 5'-*O*-*β*-D-glucopyranoside (**20**), *α*-terpinyl 8-*O*-*β*-D-glucopyranoside (**21**), turpinionoside A (**22**), (*Z*)-3-hexenyl *O*-*α*-L-arabinopyranosyl-(1 → 6)-*β*-D-glucopyranoside (**23**), unshuoside A (**24**), rupicolin B (**25**), rupicolin A (**26**), 1*α*,4*α*-dihydroxybishopsolicepolide (**27**), 1*α*,4*α*-

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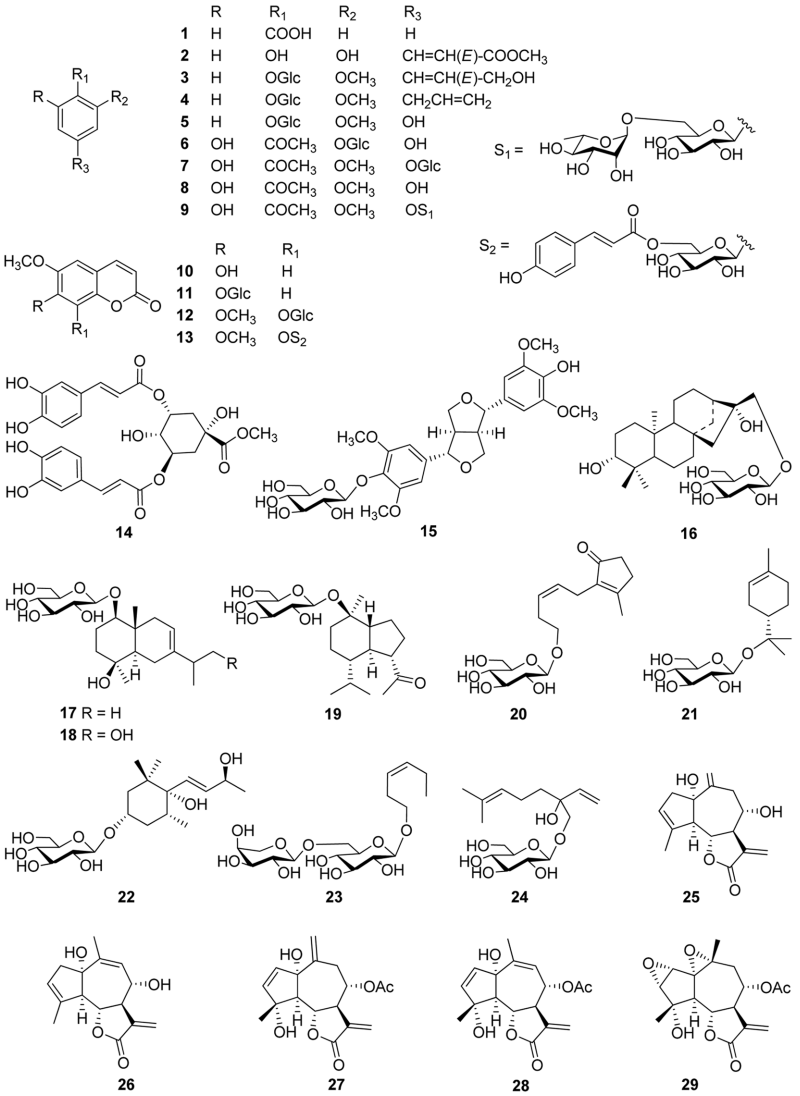

Fig. 1. Chemical structures of compounds **1** - **29**.

dihydroxy-8α-acetoxy-guaia-2,9,11(13)-trien-6,12-olide (**28**), and iwayoside B (**29**) by physical and spectroscopic characterizations (Fig. 1).[8-11]

In the present study, we examined the antioxidant effects of these 29 phytochemicals using *in vitro* and cellular assays, including the oxygen radical absorbance capacity (ORAC) test, which is one of the most popular and well-characterized antioxidant assays,[12,13] as well as reducing capacity and metal chelating tests.[14-16] 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) was used to generate peroxyl radicals in the ORAC assay. Cell culture experiments were performed using the human-derived hepatoma HepG2 cell line, which allow accurate and reliable assessment of antioxidant activity in mammalian cells.[17,18]

Experimental

General experiment procedures – AAPH, Trolox, fluorescein, calcein, neocuproine, Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), phosphate buffered saline (PBS, pH 7.4), dichloro-dihydro-fluorescein diacetate (DCFH-DA), and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The HepG2 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA).

Plant material – Aerial parts of *A. iwayomogi* were collected on Jeju Island in June 2007, and taxonomically identified by Prof. Young Ho Kim at the College of Pharmacy, Chungnam National University, Daejeon, Korea. A voucher specimen (CNU07105) has been deposited at

the herbarium of the above college.

ORAC assay – The ORAC assay was carried out using a Tecan GENios multi-functional plate reader (Salzburg, Austria) with fluorescent filters (excitation wavelength: 485 nm, emission filter: 535 nm). In the final assay mixture, fluorescein (40 nM) was used as a target of free radical attack with AAPH (20 mM) as a peroxyl radical generator in the peroxyl radical-scavenging capacity assay. The analyzer was programmed to record fluorescein fluorescence every 2 min after the addition of AAPH. All fluorescence measurements were expressed relative to the initial reading. Final values were calculated on basis of the difference in the area under the fluorescence decay curve between the blank and test samples. All data are expressed as net protection area (net area). Trolox (1 μM) was used as the positive control to scavenge peroxyl radicals.[19]

Reducing capacity – The electron-donating capacities of selected compounds to reduce Cu^{2+} to Cu^{+} were assessed according to the method of Aruoma *et al*.[20] Forty microliters of different concentrations of each compound in ethanol were mixed with 160 μL of a mixture containing 0.5 mM $CuCl_2$ and 0.75 mM neocuproine, a Cu^{+} specific chelator, in 10 mM phosphate buffer, pH 7.4. Absorbance was measured using a microplate reader at 454 nm for 1 h. Increased absorbance of the reaction mixture indicated greater reducing power.

Metal chelating activity – Metal chelating activity was measured using the competitive reaction procedure described by Argirova and Ortwerth.[21] One hundred microliters of different concentrations of each compound in ethanol were mixed with 100 μL of 0.4 μM $CuSO_4$. After 100 μL of the mixture solution was incubated with 100 μL of 0.2 μM calcein for 1 h, fluorescence of the mixture solution was measured using a Tecan GENios multi-functional plate reader with fluorescent filters (excitation wavelength: 485 nm and emission filter: 535 nm) and compared to the fluorescence intensity of the control, which contained only calcein.

Cellular antioxidant activity – Cellular oxidative stress due to ROS generated by AAPH or Cu^{2+} was measured spectrofluorometrically by the DCFH-DA method.[22] DCFH-DA diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to non-fluorescent DCFH, which can be rapidly oxidized to highly fluorescent DCF in the presence of ROS. HepG2 cells were firstly cultured in 96-well plates (5×10^5/mL) with DMEM for 24 h. After the cells were incubated with different concentrations of each compound in DMSO for 30 min, the media were discarded, and the wells were gently washed twice with PBS. Instead of normal media, HBSS, which is stable to fluorescence, was added to each well. AAPH was used to induce peroxyl radical oxidative stress, and copper (Cu^{2+}) was used to induce another type of oxidative stress. After the cells were treated with 60 μM AAPH or 10 μM Cu^{2+} for 30 min, DCFH-DA was added to the culture plates at a final concentration of 40 μM and incubated for 30 min at 37 °C in the dark. DCF fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a Tecan GENios fluorometric plate reader.

Statistical analysis – All data are presented as means ± standard deviations (SD). Statistical analysis was carried out using the IBM SPSS statistical package (Version 21.0, IBM, New York, USA) program, and the significance of each group was verified with a one-way analysis of variance (ANOVA) followed by Duncan's test. A p value < 0.05 was considered significant.

Results and Discussion

In vitro* antioxidant activities of compounds from *A. iwayomogi – Antioxidant activities of compounds **1 - 29** from *A. iwayomogi* were initially evaluated for their peroxyl radical-scavenging capacities employing an ORAC assay, in which AAPH, an azo compound, was used to generate peroxyl radicals. As shown in Table 1, compounds **2**, **6**, **8**, **10**, **13**, and **14** showed potent and dose-dependent peroxyl radical-scavenging activities at 1 - 10 μM. Structural analysis of the active phytochemicals suggested that phenolic hydroxyl groups were responsible for the peroxyl radical-scavenging activity; these groups might donate hydrogen atoms. The ability of compounds **2**, **6**, **8**, **10**, **13**, and **14** to stimulate the reduction of Cu^{2+} to Cu^{+} was then investigated to determine whether their strong peroxyl radical-scavenging capacities resulted from electron donation to peroxyl radicals. As shown in Fig. 2, compounds **2**, **10**, and **14** showed greater reducing capacities than did **6**, **8**, and **13** at 1 - 10 μM. This observation indicates that two hydroxyl groups at *ortho* positions of the aromatic ring (compounds **2** and **14**) or a hydroxyl group at the C-7 position of the coumarin (**10**) could be responsible for electron transfer to the Cu^{2+} ion. Furthermore, our data suggest that the peroxyl radical-scavenging capacities of compounds **2**, **10**, and **14** may be attributable to reduction of peroxyl radicals through donation of electrons or hydrogens. Another mechanism by which natural products can inhibit the generation of hydroxyl radicals is chelation of metal ions, which prevents their interaction with hydrogen peroxide. Therefore,

Table 1. Peroxyl radical-scavenging activities of compounds **1** - **29**.

Compound	Peroxyl radical-scavenging capacity (TE, μM)	
	1 μM	10 μM
1	–0.45 ± 0.11	0.33 ± 0.13
2	2.82 ± 0.12	12.99 ± 0.65
3	0.52 ± 0.22	2.73 ± 0.37
4	0.64 ± 0.09	3.14 ± 0.15
5	1.07 ± 0.22	5.38 ± 0.36
6	2.66 ± 0.22	11.74 ± 0.69
7	0.19 ± 0.16	2.27 ± 0.35
8	3.29 ± 0.53	9.18 ± 0.40
9	0.87 ± 0.30	2.79 ± 0.19
10	2.38 ± 0.11	10.44 ± 0.60
11	0.22 ± 0.10	1.56 ± 0.28
12	0.05 ± 0.22	0.38 ± 0.40
13	4.25 ± 0.36	17.72 ± 0.87
14	2.17 ± 0.22	12.66 ± 0.61
15	1.43 ± 0.18	6.27 ± 0.29
16	–0.38 ± 0.24	0.05 ± 0.03
17	0.60 ± 0.15	1.88 ± 0.14
18	0.41 ± 0.15	2.18 ± 0.21
19	0.44 ± 0.10	0.82 ± 0.17
20	1.00 ± 0.16	4.69 ± 0.22
21	0.55 ± 0.22	2.53 ± 0.18
22	0.39 ± 0.20	1.90 ± 0.16
23	0.24 ± 0.13	1.36 ± 0.28
24	0.44 ± 0.07	1.84 ± 0.14
25	0.75 ± 0.50	1.95 ± 0.33
26	0.05 ± 0.10	3.06 ± 0.08
27	–0.15 ± 0.19	2.23 ± 0.22
28	0.51 ± 0.11	4.18 ± 0.50
29	–0.02 ± 0.15	0.72 ± 0.18

All data are expressed as the mean ± SD from three individual experiments. Values are expressed as μM of Trolox equivalents (TE); one ORAC unit is equivalent to the net protection area provided by 1 μM of Trolox.

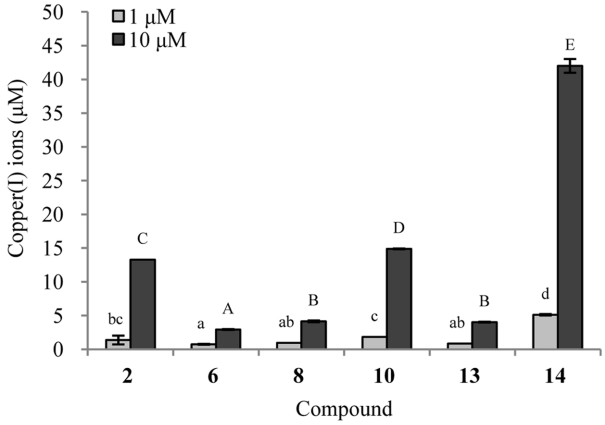

Fig. 2. Reducing capacities of selected compounds from *A. iwayomogi.* Results are expressed as mean ± SD from three individual experiments and values with different letters at the same concentration indicate significant differences ($p < 0.05$).

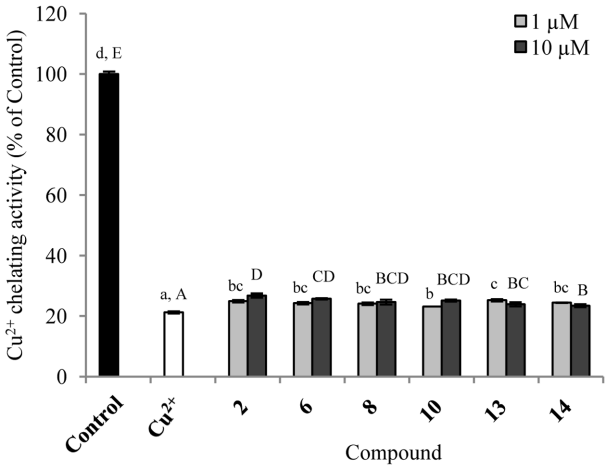

Fig. 3. Metal chelating activities of selected compounds from *A. iwayomogi*. Percentages are relative to the value obtained from control that did not contain copper ions. Results are expressed as means ± SD from three individual experiments and values with different letters at the same concentration indicate significant differences ($p < 0.05$).

the Cu^{2+} chelating capacities of compounds **2**, **6**, **8**, **10**, **13**, and **14** were analyzed using calcein, a fluorescent probe that loses its fluorescence upon formation of the cupric ion complex. The fluorescence generated by each compound was proportional to its chelating activity with cupric ions. As shown in Fig. 3, all tested compounds showed weak metal chelating activities at 1 - 10 μM. This result suggests that the antioxidant activity of these compounds does not involve chelation of transition metal ions.

Cellular antioxidant activities of compounds from *A. iwayomogi* – The cellular antioxidant capacities of compounds **2**, **6**, **8**, **10**, **13**, and **14** were investigated using a cellular antioxidant capacity assay. HepG2 cells were pre-incubated with the selected compounds (1 - 10 μM) for 30 min. After the incubation, the cells were exposed to 60 μM AAPH or 10 μM Cu^{2+} for 30 min and then treated with DCFH-DA, a fluorescent probe that detects ROS, for 30 min to measure the oxidative stress induced by AAPH or Cu^{2+}. As shown in Fig. 4A and 4B, the intracellular oxidative stress in HepG2 cells increased to 149.55% and 175.44% following treatments with AAPH and Cu^{2+}, respectively, compared to untreated cells (the control group). All selected compounds decreased the cellular oxidative stress caused by AAPH and Cu^{2+} at 1 - 10 μM, and

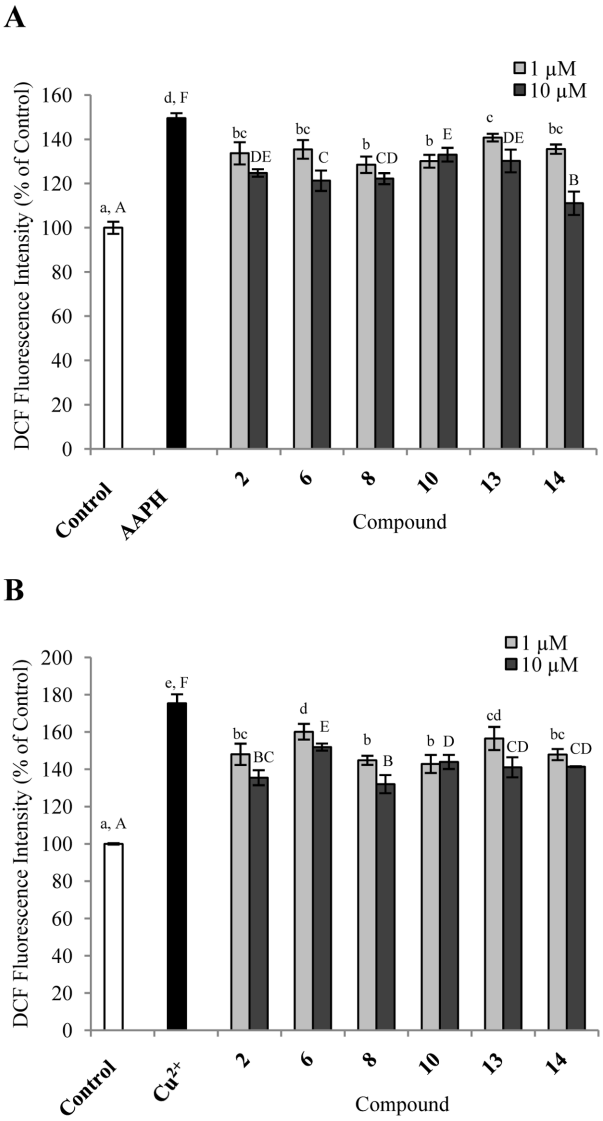

Fig. 4. Cellular antioxidant capacities of selected compounds from *A. iwayomogi* against oxidative stress induced by AAPH (A) and Cu^{2+} (B). Percentages are relative to the values obtained from untreated cells. Results are expressed as means ± SD from three individual experiments and values with different letters at the same concentration indicate significant differences ($p < 0.05$).

compounds **2**, **6**, **8**, **13**, and **14** reduced the extent of cellular oxidative stress in a dose-dependent manner (Fig. 4). At 10 μM, compounds **6**, **8**, and **14** scavenged more peroxyl radicals generated by AAPH than did the other compounds, and reduced the oxidative stress to 121.28%, 122.23%, and 111.10%, respectively (Fig. 4A). In addition, compounds **2** and **8** showed greater antioxidant capacities against the oxidative stress induced by Cu^{2+} than did the other compounds at 10 μM (135.47% and 132.03%, respectively; Fig. 4B).

These results differ from the antioxidant activities of the tested compounds in *in vitro* assay, and this difference was suggested to be derived from the different cell membrane permeability of the tested compounds. Further investigations into cellular antioxidant metabolism are required to test this hypothesis. In summary, we conclude that the phenolic compounds, especially those containing multiple hydroxyl groups, were the major antioxidant constituents of *A. iwayomogi* and are responsible for the potent antioxidant activity of the *A. iwayomogi* extract.

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